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# The Membrane-Bound Spermatozoal Adenylyl Cyclase System Does not Share Coupling Characteristics with Somatic Cell Adenylyl Cyclases*

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**ABSTRACT.** Membrane-bound adenylyl cyclases from ram, dog, and human sperm are unresponsive to fluoride and guanylylimidodiphosphate [GMP-P(NH)P], two agents that stimulate the adenylyl cyclases of somatic cells by an action on the stimulatory guanine nucleotide-binding regulatory ($N_s$) component of adenylyl cyclase. We have investigated whether this is because the sperm cell catalytic unit is functionally uncoupled from $N_s$ but, nevertheless, capable of interacting with it, or because the sperm cell adenylyl cyclase system is unique and regulated differently from that of somatic cells. Sperm cells were found to be deficient in $N_s$, as evidenced by the inability of detergent extracts from sperm cell membranes and fractions to reconstitute $N_s$-mediated regulation of the adenylyl cyclase of $cyc^-$ S49 cells. In addition, attempts to label $N_s$ in sperm cell membranes by [$^{32}$P]ADP ribosylation with cholera toxin revealed that, if present, $N_s$ is less than 1% of that found in human erythrocyte membranes. This, however, was not the only reason for the unresponsiveness of sperm cell adenylyl cyclase, since fluoride stimulation of the sperm cell enzyme could not be induced by reconstituting it with $N_s$ purified from human erythrocytes (hRBC). When intact hRBC membranes were added to sperm cell fractions in the presence of fluoride, the activities that resulted were greater than the sum of the individual activities. This apparent reconstitution of fluoride regulation of sperm cell adenylyl cyclase could be blocked by lima bean trypsin inhibitor and appears to have resulted from proteolytic activation of the hRBC adenylyl cyclase by sperm proteases. Sperm cell membranes also appear to lack a functional inhibitory regulatory protein of the adenylyl cyclase system ($N_i$), since they did not contain an ADP-ribosylatable substrate for pertussis toxin action. These results suggest that the sperm cell adenylyl cyclase system is unique and different from that of somatic cells. Sperm cells appear to neither contain $N_s$ or $N_i$ nor possess the ability of their adenylyl cyclase system to interact with $N_s$ from an exogenous source. (*Endocrinology* **116:** 1357–1366, 1985)

IN SOMATIC cells, adenylyl cyclases are membrane-bound enzyme complexes, formed of multiple components, which are regulated both positively and negatively by hormones and guanine nucleotides (1–4). The catalytic component ($C$) of the system synthesizes cAMP from ATP. Isolated from the other components of the complex, $C$ is unaffected by most regulatory ligands and, in fact, exhibits high catalytic activity only in the presence of $Mn^{+2}$ and not in the presence of $Mg^{+2}$, its physiological regulator. Stimulatory and inhibitory effects on adenylyl cyclase are mediated by guanine nucleotide-binding regulatory components, $N_s$ and $N_i$, respectively. Both have been purified to homogeneity (5–8) and are the mediators of hormonal regulation of the system as well as the sites of action of guanine nucleotides, fluoride, and bacterial toxins (1–8).

Mammalian spermatids and spermatozoa contain a high level of adenylyl cyclase activity (9–11) which appears to differ from that in somatic cells in at least two important ways: 1) it is mostly soluble rather than membrane bound in spermatids, becoming membrane bound during the final stages of differentiation into spermatozoa; and 2) in both spermatids and spermatozoa, it behaves like an activity that is uncoupled from the regulatory components of adenylyl cyclase. Thus, it is very active when assayed with 5–10 mM $MnCl_2$, is much less active when assayed with 5–10 mM $MgCl_2$, and is unaf-

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fected by guanine nucleotides or fluoride ion. Hormonal regulation of the sperm cell adenylyl cyclase has not been demonstrated, and the physiological role of this enzyme is unclear, although an increase in its activity is associated with increased sperm motility (10).

Originally, we felt that the properties of the sperm adenylyl cyclase system resulted merely from the fact that it was uncoupled from the regulatory proteins of the complex. We, like others (12–14), therefore, hoped to use the sperm system to identify additional components of intact systems using standard reconstitution techniques originally developed for the identification of $N_s$ (15). The viability of this approach was suggested by the findings of Stengel and Hanoune (16) that sperm membranes added to erythrocyte membranes yielded superadditive cyclase activities which were interpreted to indicate reconstitution of the sperm system. However, when we tried to reconstitute the sperm adenylyl cyclase with purified $N_s$ protein we were unable to do so. This led us to question whether, in fact, the sperm adenylyl cyclase is the same as that of other cells. To try and find support for the idea that it might be related, we explored whether we could find evidence for the presence in sperm of the other components of somatic cell adenylyl cyclase systems, *i.e.* $N_s$ and $N_i$. As reported below, we have found no compelling evidence for the existence of these components in sperm. In addition, our results, as well as those of others published while this manuscript was in preparation (12–14), indicate that the superadditive effects of mixing sperm and erythrocyte membrane result from the proteolytic activation of the erythrocyte adenylyl cyclase. Thus, all known properties of the sperm adenylyl cyclase system suggest that it is a unique enzyme, probably with its own regulatory mechanisms which are different from those of somatic cells.

## Materials and Methods

### *Materials*

[$\alpha$-$^{32}$P]ATP was provided by the Core Laboratory on Molecular Endocrinology of the Baylor College of Medicine, Diabetes and Endocrinology Research Center (Dr. James B. Field, Director). [$^{32}$P]NAD was synthesized from [$\alpha$-$^{32}$P]ATP by the method of Cassel and Pfeuffer (17). Guanylylimidodiphosphate [GMP-P(NH)P] was purchased from Boehringer Manneheim (New York, NY) and used after purification by chromatography over DEAE-Sephadex-A25 (18). Lima bean trypsin inhibitor was purchased from Worthington Biochemical Corp. (Freehold, NJ). Cholera toxin was obtained from List Laboratories (Campbell, CA) and pertussis toxin was purified as described previously (19). All other reagents and biochemicals were of the highest commercially available grade and were used without further purification.

### *Sources of sperm*

Dog sperm free of cytoplasmic droplet contamination was obtained from ejaculated semen. After collection, it was diluted in 10 mM HEPES (pH 7.5) containing 100 mM NaCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 2 mM EGTA and frozen at −196 C until used. Ram sperm was obtained from Dr. Vidar Hansson (Agricultural Station, University of Oslo, Oslo, Norway), diluted 10 times in 50% egg yolk, kept at 0–4 C for 14 h, and processed without prior freezing. Before homogenization and subcellular fractionation, sperm cells were washed three times with 150 mM NaCl-20 mM Tris-HCl (pH 7.6) by centrifugation at 5000 rpm for 10 min in the SS-34 rotor of the RC-5 Sorvall refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, CT). Human sperm was obtained from healthy donors, was allowed to liquify, and was used either immediately or after freezing at −196 C. Sperm cells were washed, homogenized, and fractionated as described for the individual experiments reported in the text.

### *Sources of other membranes and membrane components*

*Cyc*$^-$ S49 murine lymphoma cells were grown as described by Bourne *et al.* (20), and membranes deficient in $N_s$ were prepared as described by Ross *et al.* (21), except that $MgCl_2$ was omitted from all buffers. Human erythrocyte membranes were prepared according to the method of Dodge *et al.* (22). Human erythrocyte stimulatory regulatory component was purified to better than 90% purity as described by Codina *et al.* (6). The preparation used was dissolved in 0.1% Lubrol, 50 mM NaCl, 30% ethylene glycol, 20 mM 2-mercaptoethanol, and 25 mM Na-HEPES, pH 8.0, and was 5,000-fold purified with respect to washed human erythrocyte membranes. It contained two major polypeptides of apparent mol wt of 42,000 and 35,000, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The preparation of rat liver membranes has been described previously (23). Highly luteinized ovarian membranes, comprised largely of corpus luteum membranes, were prepared from whole ovaries of superovulated rats. Thirty-day-old female rats were injected with 50 IU PMSG at 1000 h and with 50 IU hCG at 1600 h on day 32 of age. Ovaries were removed, and membranes were prepared on day 39 of age as described previously (24) and are referred to in the text as luteal membranes for the sake of brevity.

Unless indicated otherwise, $N_s$ activity was extracted from sperm, luteal, liver, or erythrocyte membranes by adding 20 $\mu$l 10% Tris-cholate (pH 8.0) to 200 $\mu$l membrane suspension (final cholate concentration, 0.91%) at a membrane protein concentration of 5 mg/ml. Samples were allowed to stand at 0–4 C for 60 min with occasional stirring and then centrifuged at 100,000 × $g$ for 60 min. Supernates containing the extracted $N_s$ activity were removed with a pipet and used immediately in assays for the reconstitution of *cyc*$^-$ S49 cell membrane adenylyl cyclase activity (see below).

### *Adenylyl cyclase assays*

Unless indicated otherwise, membranes were incubated for 40 min at 32.5 C in 50 $\mu$l medium containing 0.1 mM [$\alpha$-$^{32}$P]

TABLE 1. Adenylyl cyclase activities in human, dog, and ram sperm cells and lack of effect of NaF

| Membrane fraction assayed | Protein added to assays | Adenylyl cyclase activities (pmol/min·mg) | | |
|---|---|---|---|---|
| | | $MnCl_2$ | $MgCl_2$ | |
| | | | — | NaF |
| A. Ram sperm[a] | | | | |
| 1. Procedure A | | | | |
| Homogenate | 1.3 μg | 34.4 ± 0.4 | | 1.28 ± 0.38 |
| 0–6,000 × *g* pellet | 1.5 μg | 57.2 ± 2.4 | | 1.55 ± 0.22 |
| 6,000–100,000 × *g* pellet | 1.3 μg | 120.9 ± 5.2 | | 3.58 ± 0.39 |
| 2. Procedure B | | | | |
| Homogenate | 1.0 μg | 38.8 ± 1.5 | | 1.50 ± 0.33 |
| 0–250 × *g* pellet | 6.5 μg | 8.8 ± 0.5 | | 0.30 ± 0.05 |
| 250–6,000 × *g* pellet | 3.5 μg | 27.4 ± 3.2 | | 0.71 ± 0.08 |
| 6,000–100,000 × *g* pellet | 0.8 μg | 254 ± 9.8 | | 5.29 ± 0.23 |
| B. Dog sperm (procedure B)[b] | | | | |
| 0–6,000 × *g* pellet | 7.0 μg | 12.2 ± 0.1 | 0.52 ± 0.17 | 0.40 ± 0.15 |
| 6,000–100,000 × *g* pellet | 1.1 μg | 323 ± 2.3 | 20.1 ± 4.0 | 16.0 ± 2.0 |
| C. Human sperm (procedure A)[c] | | | | |
| 1. Fresh sperm | | | | |
| 0–45,000 × *g* pellet | 4.2 μg | 11.5 ± 0.3 | 2.73 ± 0.59 | 2.92 ± 0.06 |
| 2. Frozen sperm | | | | |
| 0–45,000 × *g* pellet | 2.6 μg | 15.8 ± 0.2 | 2.50 ± 0.09 | 2.41 ± 0.11 |

[a] Ram sperm ejaculates (6 ml in 30 ml egg yolk medium) were washed three times with 80 ml 150 mM NaCl-10 mM Tris-HCl, pH 7.5 (5,000 × *g* for 10 min at 4 C). The washed sperm cells were resuspended in 100 ml wash buffer and subjected to homogenization and fractionation according to two procedures. Procedure A, Twenty-five milliliters of resuspended and washed sperm cells were centrifuged for 10 min at 6,000 × *g* in the cold, resuspended in 20 ml 1 mM $KHCO_3$, homogenized (32), and centrifuged for 20 min at 6,000 × *g*. The pellet (low speed pellet) was resuspended in 1 mM $KHCO_3$ and assayed. The supernatant was centrifuged at 45,000 × *g* for 60 min. The pellet (high speed pellet) was resuspended and assayed. Procedure B, Seventy-five milliliters of resuspended and washed sperm cells were homogenized by $N_2$ cavitation (40) after a 15-min equilibration at 700 psi. The resulting homogenate was centrifuged first at 250 × *g* for 5 min to separate sperm heads stripped of their plasma membranes, then at 6,000 × *g* for 20 min, yielding a sediment rich in tails and membrane fragmants, and finally at 100,000 × *g* for 60 min, yielding a pellet formed of membrane sheets and vesicles. The pellets from each centrifugation were resuspended in 1 mM $KHCO_3$ and assayed.

[b] Dog sperm cells were pelleted and resuspended in 150 mM NaCl, 1 mM EDTA, and 20 mM Tris-HCl, pH 7.6. The mixture was homogenized by $N_2$ cavitation (40) after equilibration at 700 psi for 15 min. The homogenate was centrifuged first at 6,000 × *g* for 20 min and then at 100,000 × *g* for 60 min, yielding low and high speed pellets, respectively. The pellets were resuspended in 1 mM $KHCO_3$ and assayed.

[c] Ejaculates (fresh or frozen in liquid $N_2$) were washed twice with 20 vol 150 mM NaCl and 1 mM $NaHCO_3$ by centrifugation at 750 × *g* at 4 C for 20 min. The pellets were homogenized (16) in 2 ml 1 mM $NaHCO_3$, centrifuged at 45,000 × *g* for 10 min at 4 C, resuspended in 1 mM $NaHCO_3$, and assayed.

ATP (~2000 cpm/pmol), 10 mM $MgCl_2$ or 10 mM $MnCl_2$, 1.0 mM EDTA, 1.0 mM [$^3$H]cAMP (~10,000 cpm/assay), when present 10 mM NaF or 10 μM GMP-P(NH)P, and a nucleoside triphosphate-regenerating system consisting of 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, and 0.02 mg/ml myokinase, and 25 mM Tris-HCl, pH 7.6. Reactions were stopped, and the [$^{32}$P]cAMP formed was assayed by the method of Salomon *et al.* (25), as modified by Bockaert *et al.* (26).

*Reconstitution assays and complementation attempts*

$N_s$ activity in detergent (cholate) extracts from human erythrocyte, rat luteal, rat liver, or sperm cell membranes was assayed by the reconstitution of $N_s$-mediated regulation of the adenylyl cyclase of *cyc*$^-$ S49 cell membranes (15). Stimulation of the enzyme in *cyc*$^-$ membranes by GMP-P(NH)P or NaF was taken as evidence that the system had been reconstituted. *Cyc*$^-$ membranes (10–30 μg protein) in 10 μl were mixed with 10 μl of an appropriate dilution (see individual experiments) of cholate extract from membranes or of purified $N_s$ and kept on ice for 15–20 min. Control incubations, measuring the endogenous *cyc*$^-$ activity, contained 10 μl *cyc*$^-$ membranes and 10 μl equivalently diluted detergent. Adenylyl cyclase activities were assayed by the postaddition of 30 μl reagents to produce the assay conditions described above for adenylyl cyclase assays, with 10 mM $MgCl_2$ and incubation for 40 min at 32.5 C. Reactions were stopped, and the [$^{32}$P]cAMP formed was assayed as described above.

For complementation of sperm membrane adenylyl cyclase with purified $N_s$ or human erythrocyte membrane-bound $N_s$, 10 μl sperm membranes (for details, see text) were mixed with 10 μl appropriately diluted $N_s$ or human erythrocyte membranes (25–30 μg protein) and allowed to stand on ice for 15–20 min. Complementation assays were begun by the postaddition of 30 μl reagents to give the assay conditions described above for adenylyl cyclase assays in the presence of 10 mM $MgCl_2$ and either NaF or GMP-P(NH)P. After 40 min at 32.5 C, the reactions were stopped, and the [$^{32}$P]cAMP formed was quantified as described above.

*[$^{32}$P]ADP ribosylation by cholera toxin*

Cholera toxin was activated at a concentration of 2.5 mg/ml by incubation at 32 C for 30 min in the presence of 25 mM

TABLE 2. Lack of effect of highly purified human erythrocyte stimulatory regulatory component of adenylyl cyclase ($N_s$) on sperm membrane adenylyl cyclase

| Membranes added to assays | Adenylyl cyclase activities | | | | |
|---|---|---|---|---|---|
| | $MnCl_2$ (pmol cAMP/min·mg) | $MgCl_2$ (pmol cAMP/40 min) | | $MgCl_2$ plus $N_s$ (pmol cAMP/40 min)[a] | |
| | | — | NaF | — | NaF |
| $Cyc^-$ S49 cell[b] | 6 ± 1 | 0.10 ± 0.01 | 0.09 ± 0.01 | 0.36 ± 0.01 | 22.4 ± 0.8 |
| Ram sperm[c] | | | | | |
| 1. Procedure A | | | | | |
| 6,000–100,000 × *g* pellet | 167 ± 8 | 0.40 ± 0.02 | 0.34 ± 0.01 | 0.41 ± 0.04 | 0.34 ± 0.02 |
| 2. Procedure B | | | | | |
| 0–200 × *g* pellet | 16 ± 1 | 0.17 ± 0.02 | 0.15 ± 0.02 | 0.16 ± 0.01 | 0.14 ± 0.01 |
| 250–6,000 × *g* pellet | 25 ± 1 | 0.25 ± 0.03 | 0.20 ± 0.01 | 0.21 ± 0.01 | 0.18 ± 0.01 |
| 6,000–100,000 × *g* pellet | 265 ± 5 | 0.33 ± 0.02 | 0.26 ± 0.01 | 0.30 ± 0.01 | 0.26 ± 0.01 |

[a] When present, $N_s$ was 3 ng/assay.

[b] There were 16 μg $cyc^-$ membrane protein/assay.

[c] Ram sperm membranes were prepared as described in Table I. In the Procedure A fraction, there were 2.6 μg membrane protein/assay. For Procedure B, assays contained 11.5 μg membrane protein in the 0–200 × *g* samples, 6.5 μg in the 250–6,000 × *g* samples, and 2.0 μg in the 6,000–100,000 × *g* samples.

dithiothreitol. Assays for the cholera toxin substrate contained, in a final volume of 60 μl, 10 μg membrane protein, 300 mM potassium phosphate (pH 7.5), 1 mM ATP, 0.1 mM GTP, 15 mM thymidine, 1 mM EDTA, 5 mM $MgCl_2$, and $5 \times 10^6$ cpm [$^{32}$P]$NAD^+$ (~1000 Ci/mmol). Incubations were carried out for 30 min at 32 C in the presence or absence of 100 μg/ml activated cholera toxin and were stopped by adding 1 ml cold trichloroacetic acid. Protein was precipitated by centrifugation at 3000 rpm for 30 min, and the pellets were washed once with cold ethyl ether. The pellets were resuspended with 50 μl Laemmli sample buffer (27). Twenty-five microliters of each sample were electrophoresed on 10% polyacrylamide gels made with the Laemmli buffer system (27). Gels were stained with 0.1% Coomassie blue and destained with 10% acetic acid. Gels were dried between two sheets of dialysis membrane (Bio-Rad Laboratories, Richmond, CA) and autoradiographed using Kodak XR-5 film (Eastman Kodak, Rochester, NY) for 1–3 days at −70 C. Photographs of the autoradiograms are shown.

### *[$^{32}$P]ADP ribosylation with pertussis toxin*

Pertussis toxin was activated at a concentration of 0.25 mg/ml by incubation at 32 C for 30 min in the presence of 25 mM dithiothreitol. Assays for the pertussis toxin substrate contained, in a final volume of 60 μl, 10 μg membrane protein, 50 mM potassium phosphate (pH 7.5), 1 mM ATP, 0.1 mM GTP, 1 mM EDTA, 15 mM thymidine, 0.02% Lubrol PX, and $5 \times 10^6$ cpm [$^{32}$P]$NAD^+$ (~1000 Ci/mmol). Incubations were carried out for 30 min at 32 C in the presence or absence of 10 μg/ml activated pertussis toxin. Processing of the samples after this incubation was the same as that for the assays of cholera toxin substrate (see above).

### *Linearity of assays and expression of results*

Assays were carried out under conditions that were linear with respect to protein addition. Adenylyl cyclase activities in membrane fractions are expressed as picomoles of cAMP formed per mg protein/min. Reconstituted or complemented activities, while linear with respect to protein addition (be it the fraction responsible for catalytic activity or that responsible for regulatory component activity), were nonlinear with respect to time and exhibited lag times in the progress curves of cAMP accumulation ranging from 10–20 min. These lag times were inherent in the process being measured and varied with the batch of $cyc^-$ membranes used and with the concentration of $MgCl_2$ present in the assays (not shown). Due to this, reconstituted or complemented activities were expressed as picomoles of cAMP formed per 40 min without or with additional referral to the membrane protein assumed to contribute the acceptor catalytic component of the adenylyl cyclase system. All results are the mean ± SD of triplicate determinations. All experiments were carried out at least twice to ensure reproducibility. Proteins were determined by the method of Lowry *et al.* (28) using BSA as standard.

## Results

### *Adenylyl cyclase activities in various sperm cell membranes*

Adenylyl cyclase activities were tested in homogenates of ram, dog, and human sperm cells. As illustrated in Table 1, the activities were significantly higher in the animal sperm cell preparations than in those from human sperm. Regardless of the species of origin, however, sperm cell adenylyl cyclases were insensitive to either NaF or GMP-P(NH)P stimulation and were significantly higher when assayed with $MnCl_2$ than with $MgCl_2$ as the divalent cation. Thus, ratios of activities measured with $MnCl_2$ to those measured with $MgCl_2$ plus NaF ranged from 25–40 for ram sperm fractions, from 20–40 for dog sperm fractions, and from 3–7 for human sperm. These results are compatible with those of others who have investigated the properties of the sperm adenylyl cyclase system (9–11).

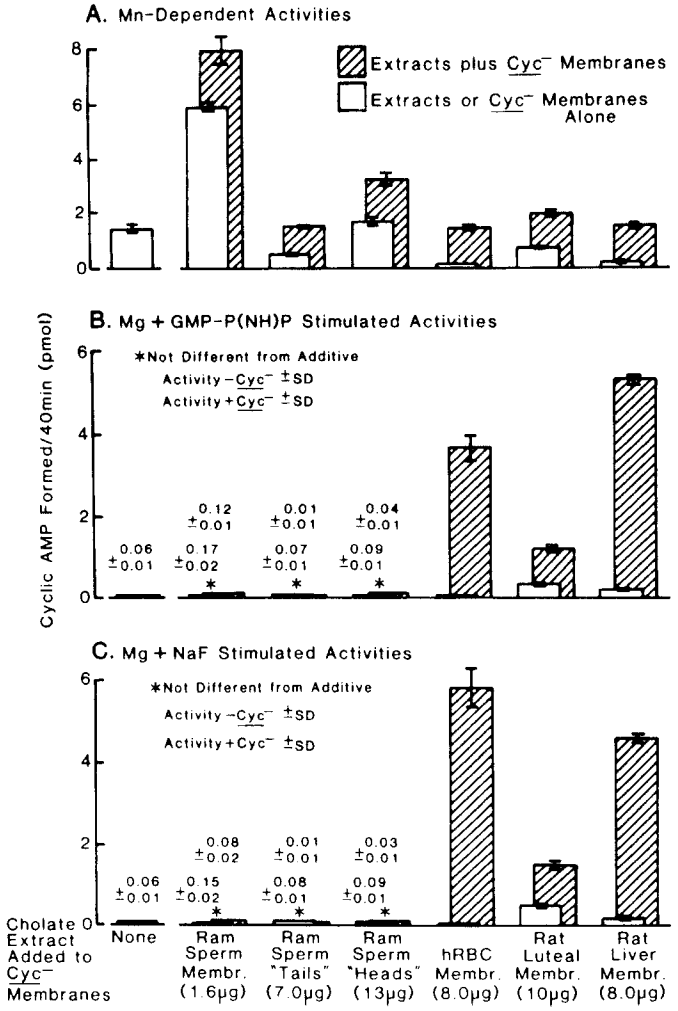


FIG. 1. Effect on adenylyl cyclase activity of combining cholate extracts from membranes of ram sperm, human erythrocytes, rabbit corpora lutea, or rat livers with *cyc*⁻ S49 cell membranes. Adenylyl cyclase activities of cholate extracts of various ram sperm fractions or human erythrocyte, rat corpus luteal, or rat liver membranes in the absence (□) or presence (▨) of 11 μg *cyc*⁻ S49 cell membrane protein. The *lefthand open bars* represent the activities of *cyc*⁻ membranes alone in the presence of 0.036% cholate, the concentration of the detergent contributed to the assay by the extracts. The assay conditions are those given in *Materials and Methods*, except that the incubation medium contained 1 mg/ml lima bean trypsin inhibitor (see text for explanation). A, Activities in the presence of 10 mM $MnCl_2$. B, Reconstituted activities assayed with 10 μM GMP-P(NH)P and 10 mM $MgCl_2$. C, Reconstituted activities assayed with 10 mM NaF and 10 mM $MgCl_2$. In B and C, the *upper row of numbers* represents the numerical values corresponding to the activities of the *open bars* (*i.e.* in the absence of *cyc*⁻ membranes). The *lower row of numbers* represents the numerical values corresponding to the *hatched bars* (*i.e.* in the presence of *cyc*⁻ membranes). Values at the *bottom of the figure* are the equivalent amounts of membrane protein that contributed to the cholate extracts in each sample.

### *Lack of effect of pure $N_s$ on sperm membrane adenylyl cyclase*

Ram sperm membranes with specific activities of adenylyl cyclase between 300–350 pmol cAMP formed/min·mg protein under the assay conditions used here were tested for their ability to interact with $N_s$ purified from human erythrocytes. As illustrated in Table 2, ram sperm membrane adenylyl cyclase was not significantly affected by the presence of purified $N_s$ under conditions that enhance the activity of $N_s$-deficient *cyc*⁻ S49 cell membranes in the presence of NaF by a factor of 200-fold. Similar results were obtained with dog sperm membranes or human sperm membranes as putative acceptors of $N_s$ activity (not shown). These data suggest that the membrane-bound form of sperm adenylyl cyclase, like the soluble form (11, 15), is incapable of interacting with the $N_s$ protein that regulates the activity of the enzyme of somatic cells.

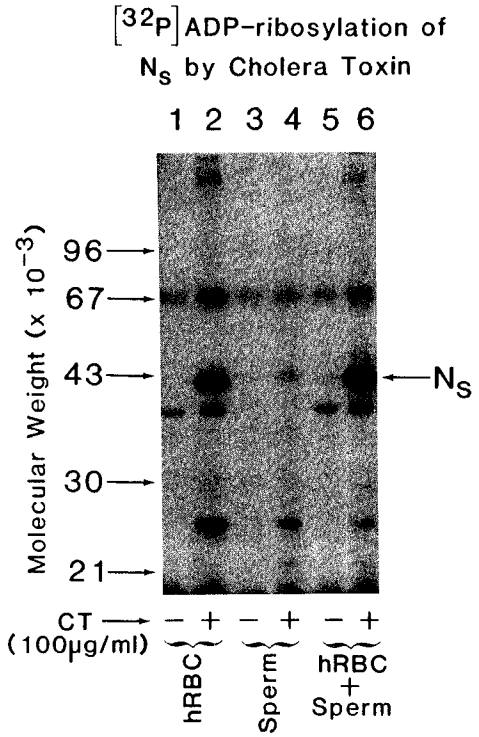


FIG. 2. [$^{32}$P]ADP ribosylation of $N_s$ by cholera toxin. Dog sperm membranes were prepared as described in Table 1. The 100,000 × *g* pellet was used in this experiment. The preparation of the human erythrocyte (hRBC) membranes and the incubation conditions for the assay are given in *Materials and Methods*. The two samples on the *right* contained 10 μg each of sperm membranes and hRBC membranes.

### *Lack of evidence for the presence of $N_s$ in sperm*

The above experiment suggested that the sperm adenylyl cyclase was unable to associate with $N_s$. This caused us to question whether the sperm system is, in fact, related to that of other cells. The only way to conclusively answer this question would be to compare the purified enzymes from sperm and other cells types. This, however, is not feasible by currently available methodology. Short of this, we sought to characterize in sperm the other component of adenylyl cyclase systems

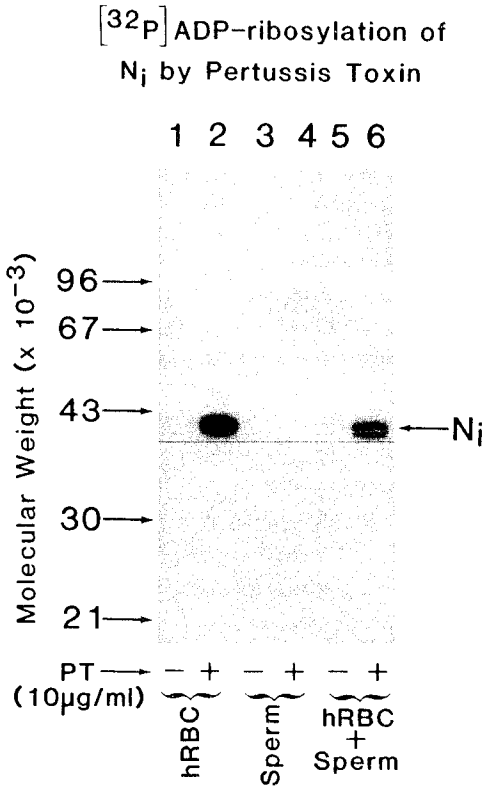


FIG. 3. [$^{32}$P]ADP ribosylation of $N_i$ by pertussis toxin. Dog sperm membranes were used, as described in Fig. 3. The two samples on the *right* contained 10 μg each of sperm membranes and human red blood cell (hRBC) membranes.

known to exist in virtually all other mammalian cells, *i.e.* $N_s$ and $N_i$.

In part, we reasoned that the possibility existed that the sperm enzyme is a precursor of a form that becomes sensitive to $N_s$ at some later stage of maturation or capacitation. If this is true, then one might expect sperm to contain measurable levels of $N_s$ activity even if at the time the sperm are isolated, the catalytic component of adenylyl cyclase is not functionally coupled to $N_s$. To test for this possibility, we used as an assay for $N_s$ activity the reconstitution of NaF- and GMP-P(NH)P-stimulated adenylyl cyclase activities in $N_s$-deficient *cyc*$^-$ S49 cell membranes (29). Detergent (cholate) extracts were prepared from sperm membranes and, as a control for the extraction procedure, from rat liver, human erythrocyte, and rat luteal membranes (see *Materials and Methods*). When these extracts were combined with *cyc*$^-$ S49 cell membranes (Fig. 1), all but those made from sperm membranes contained measurable $N_s$ activity. In fact, not only were the sperm membranes with high specific activity adenylyl cyclase deficient in measurable $N_s$, but so were other sperm fractions (composed of primarily heads or tails) which make up the bulk of the particulate material from the fraction of sperm cells (Fig. 1). The same results were found whether the sperm were extracted with cholate alone or cholate and high salt, a procedure necessary for extraction of some sources of $N_s$ (30).

A second approach for the identification of $N_s$ is by its ability to be ADP ribosylated by cholera toxin in the presence of $NAD^+$. We, therefore, looked for whether sperm cell membranes contain a cholera toxin substrate. Compared to human erythrocyte membranes, which were used as a control for the labeling procedure, sperm cell membranes contained no definitive labeling of 42,000-dalton cholera toxin substrate characteristic of $N_s$ (Fig. 2). The slight labeling of material at about 42,000 observed in sperm membranes, while it may or may not be representative of $N_s$, was determined by densitometric scanning to be less than 1% of that found in an equivalent amount of human erythrocyte membranes. In other experiments (not shown), addition of a partially purified factor that promotes ADP ribosylation by cholera toxin (provided to us by Dr. Michael Gill, Tufts University Medical School) did not improve labeling of the putative $N_s$ at 42,000 mol wt. In addition, as far as we could tell, this low apparent level of $N_s$ was not due to the fact that the sperm membranes contain some factor that interferes with the labeling procedure. Mixtures of erythrocyte and sperm membranes allowed for essentially the same extent of labeling as the erythrocyte membranes alone (Fig. 2). These results are compatible with those above, indicating that $N_s$ levels in sperm are very low, if not absent entirely.

### *Lack of evidence for presence of $N_i$ in sperm membranes*

It is now recognized that intact adenylyl cyclase systems are composed of two distinct regulatory proteins: one mediating stimulation of the enzyme ($N_s$) and the other mediating inhibition ($N_i$) (1–8). The latter of these two proteins is a substrate for ADP ribosylation by pertussis toxin (31), rather than cholera toxin. We, therefore, used pertussis toxin to try to identify $N_i$ associated with sperm membranes. Once again, human erythrocyte membranes were used as a control for the labeling procedure. Figure 3 shows that, in contrast to the erythrocyte membrane, no detectable $N_i$ was found in the sperm membrane. In this case, however, the mixture of erythrocyte and sperm membranes contained less labeled $N_i$ than did the erythrocyte membranes alone. Such a decrease in labeling in the mixture of the two membranes was consistently observed in three experiments. The significance of this is unclear, but this may be related to the presence of a factor from sperm that interferes with inhibitory regulation of other adenylyl cyclases (12–14). Whether the lack of measurable $N_i$ in the sperm membrane itself results from the direct presence of this factor

TABLE 3. Effect of combining sperm and human erythrocyte membrane cholate extracts with *cyc*⁻ S49 cell membranes

| Source of extract or membranes[a] | Adenylyl cyclase activities (pmol/40 min)[b] | | | |
|---|---|---|---|---|
| | First extraction[c] | | Second extraction[d] | |
| | $MgCl_2$ | $MgCl_2$ + NaF | $MgCl_2$ | $MgCl_2$ + NaF |
| Erythrocytes | 0.02 ± 0.01 | 0.02 ± 0.01 | 0.01 ± 0.01 | 0.04 ± 0.03 |
| Erythrocytes + *cyc*⁻[e] | 0.20 ± 0.01 | 12.33 ± 1.09[f] | 0.16 ± 0.01 | 0.95 ± 0.04[f] |
| Sperm membranes | 0.16 ± 0.01 | 0.16 ± 0.03 | 0.17 ± 0.03 | 0.11 ± 0.01 |
| Sperm membranes + *cyc*⁻ | 0.36 ± 0.02 | 0.32 ± 0.01 | 0.35 ± 0.01 | 0.30 ± 0.01 |
| Sperm heads | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.02 ± 0.01 | 0.03 ± 0.01 |
| Sperm heads + *cyc*⁻ | 0.18 ± 0.02 | 0.16 ± 0.01 | 0.16 ± 0.01 | 0.15 ± 0.01 |
| Sperm tails | 0.05 ± 0.01 | 0.04 ± 0.01 | 0.02 ± 0.01 | 0.02 ± 0.01 |
| Sperm tails + *cyc*⁻ | 0.20 ± 0.01 | 0.18 ± 0.01 | 0.17 ± 0.01 | 0.16 ± 0.01 |

[a] The ram sperm fractions are those from procedure B described in Table 1 and correspond to the 0–250 × *g* (heads), 250–6,000 × *g* (tails), and 6,000–100,000 × *g* (membranes) pellets. They and the human erythrocyte membranes were extracted at a concentration of 5 mg/ml membrane protein, and all were assayed at an extract concentration equivalent to 10 μg starting membrane protein/assay.

[b] Adenylyl cyclase assays were carried out under the conditions described in Fig. 1 in the presence of 10 mM $MgCl_2$ or 10 mM $MgCl_2$ and 10 mM NaF.

[c] The first extraction of the membranes was with 1% cholate, as described in *Materials and Methods.*

[d] For the second extraction, the 100,000 × *g* pellet from the first extraction was resuspended in the same final volume with 1% cholate, 300 mM NaCl, and 10 mM $MgCl_2$.

[e] The *cyc*⁻ S49 membrane protein concentration was 16 μg/assay. The adenylyl cyclase activities for the *cyc*⁻ membranes alone were 0.17 ± 0.01 and 0.16 ± 0.01 pmol/40 min in the absence and presence of NaF, respectively.

[f] Activities higher than expected from simple addition.

TABLE 4. Effect of combining sperm membrane fractions with human erythrocyte membranes on resulting adenylyl cyclase activities

| Source of membranes (protein of each added)[a] | Adenylyl cyclase activites | | | |
|---|---|---|---|---|
| | $MnCl_2$ | $MgCl_2$ | | |
| | | — | GMP-P(NH)P | NaF |
| A. Human sperm plus hRBC (pmol cAMP formed/40 min) | | | | |
| hRBC (26 μg) | 2.03 ± 0.07 | 0.19 ± 0.01 | 2.10 ± 0.06 | 7.34 ± 0.29 |
| Fresh sperm (4 μg) | 1.93 ± 0.05 | 0.41 ± 0.01 | 0.57 ± 0.04 | 0.49 ± 0.01 |
| hRBC (26 μg) + fresh sperm (4 μg) | 3.16 ± 0.08 | 0.59 ± 0.02 | 2.24 ± 0.09 | 9.84 ± 0.16[b] |
| Frozen sperm (3 μg) | 1.65 ± 0.02 | 0.26 ± 0.01 | 0.30 ± 0.01 | 0.25 ± 0.01 |
| hRBC (26 μg) + Frozen sperm (3 μg) | 3.43 ± 0.14 | 0.53 ± 0.01 | 3.49 ± 0.05[b] | 10.7 ± 0.1[b] |
| B. Ram sperm and hRBC (pmol cAMP formed/60 min) | | | | |
| hRBC (32 μg) | | | | 3.79 ± 0.28 |
| Intact washed sperm (5 μg) | 2.18 ± 0.05 | | | 0.08 ± 0.02 |
| hRBC (32 μg) + intact washed sperm (5 μg) | | | | 7.76 ± 0.25[b] |
| Procedure A membranes (1.2 μg) | 9.43 ± 0.41 | | | 0.28 ± 0.03 |
| hRBC (32 μg) + procedure A membranes (1.2 μg) | | | | 7.03 ± 0.19[b] |
| Procedure B membranes (0.85 μg) | 13.09 ± 2.34 | | | 0.31 ± 0.02 |
| hRBC (32 μg) + procedure B membranes (0.85 μg) | | | | 5.77 ± 0.23[b] |

hRBC, Human red blood cells.

[a] Sperm membrane fractions were obtained as described in Table 1.

[b] Activities that are higher than expected from simple additivity.

or from the fact that there is no $N_i$ present cannot be determined. In either case, however, there is no detectable $N_i$ present in these membranes.

*Effect of combining human erythrocyte membranes with sperm membranes*

In spite of the failure to confer guanine nucleotide or NaF sensitivity to sperm membranes by addition of pure $N_s$ from human erythrocytes, when unextracted erythrocyte membranes were mixed with sperm membranes, the NaF-stimulated activity that resulted was more than that expected from addition of the individually added activities. This is illustrated for mixtures of ram or human sperm membranes with human erythrocyte membranes in Table 4 and is compatible with the findings of Stengel and Hanoune (16) as well as those of others (12–

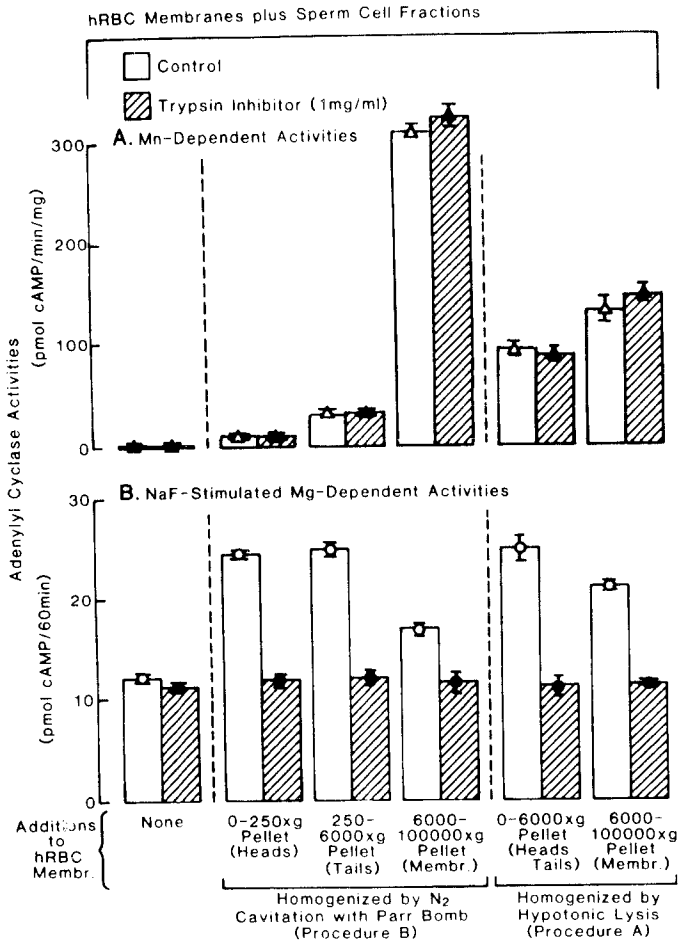


FIG. 4. Effect of trypsin inhibitor on superadditivity of adenylyl cyclase activities obtained upon mixing ram sperm membrane fractions with human red blood cell (hRBC) membranes. Ram sperm membrane fractions were prepared according to procedures A and B as described in Table 1. Membranes were assayed for adenylyl cyclase activity, as described in *Materials and Methods*, using either 10 mM $MnCl_2$ as divalent cation (A) or 10 mM $MgCl_2$ as divalent cation in combination with 10 mM NaF. ▭, Controls; ▨, incubations carried out in the presence of 1 mg/ml lima bean trypsin inhibitor. Human erythrocyte membranes were 75 μg protein/assay, and when present, the 0–250 × *g*, 250–6,000 × *g*, and 6,000–100,000 × *g* procedure B fractions were 6.5, 3.5, and 0.5 μg protein/assay, respectively, and the 0–6,000 × *g* and 6,000–100,000 × *g* procedure A fractions were 4.0 and 1.3 μg protein/assay, respectively. For the remainder of conditions, see the figure and *Materials and Methods*.

14). Although this may appear to resemble reconstitution of the sperm adenylyl cyclase system with $N_s$, such superadditivity was observed not only when various sperm membranes were mixed with human erythrocyte membranes, but also when unhomogenized sperm cells were used in the assay (Table 4).

The finding that superadditivity did not require homogenization of sperm cells raised the possibility that the phenomenon might be one of proteolytic stimulation of human erythrocyte membrane adenylyl cyclase by sperm proteases. Proteolytic activation of adenylyl cyclase activity has been observed in many systems (32–34) and has been reviewed by Lacombe *et al.* (35). To test if superadditivity was due to proteolytic activation of adenylyl cyclase, we assayed the mixtures of sperm fractions and human erythrocyte membranes in the absence and presence of lima bean trypsin inhibitor. As illustrated in Fig. 4, addition of 1 mg/ml lima bean trypsin inhibitor, which by itself affected neither the activity of sperm cell fractions nor the activity of human erythrocyte membranes, completely abolished the increase in total activity observed upon mixing sperm cell membrane fractions with human erythrocyte membranes.

## Discussion

The new findings presented here, *i.e.* the failure of pure $N_s$ to reconstitute membrane-bound sperm adenylyl cyclase, the failure to find measurable levels of $N_s$ activity in detergent extracts from sperm, and the low or undetectable levels of either cholera toxin or pertussis toxin substrate in these membranes, suggest that the sperm cell adenylyl cyclase system is unique and different from that of somatic cells. These findings should be accepted with caution and only in light of the sensitivity of the techniques that we now have available for determining the presence of $N_s$ and $N_i$ and recognizing that we have not exhausted every possible means of extracting $N_s$ activity or labeling $N_s$ and $N_i$ with bacterial toxins. These results, however, are entirely consistent with the properties of this system found by others. The high Mn-dependent (compared to Mg-dependent) activity of sperm cell adenylyl cyclase (9–11), its failure to respond to direct hormonal regulation or guanine nucleotides (10), and its lack of response to the diterpine forskolin (36, 37), which stimulates nearly all other membrane-bound adenylyl cyclases (38), all suggest that the sperm enzyme is different from that of other cells. It is quite possible, therefore, that sperm adenylyl cyclase is not regulated directly by hormones at all, but only secondarily in response to changes in $Ca^{+2}$ levels (reviewed in Ref. 10) or other ion gradients across the sperm cell membrane (39). Conversely, the sperm adenylyl cyclase may be regulated directly by hormone receptors, but by some unconventional mechanism that does not involve the regulatory proteins $N_s$ and $N_i$.

Stengel and Hanoune (16) have observed superadditivity of the adenylyl cyclase activities of erythrocyte and sperm membranes when they are assayed together. They interpreted these results as being consistent with the idea that the regulation of the sperm cell adenylyl cyclase by guanine nucleotides can be reconstituted with an exogenously added source of $N_s$. This interpretation would be at odds with the one that we suggest here, *i.e.*

that the sperm enzyme is regulated differently from the somatic cell enzyme. We, too, observed superadditivity of the adenylyl cyclase activities of sperm and erythrocyte membranes. Using purified $N_s$, however, we were unable to substantiate the idea that exogenously added $N_s$ can associate with the sperm enzyme. In searching for an explanation for the findings of Stengel and Hanoune (16), we found that the superadditivity phenomenon could be suppressed by the addition of a protease inhibitor. This suggests that the higher cyclase activity resulted from proteolytic activation of the erythrocyte enzyme and not from reconstitution of the sperm enzyme. While this paper was in preparation, several other papers have substantiated this conclusion (12–14). All of our results, the results of recently published papers (12–14), as well as previous findings (10) are entirely in line with our conclusion that the sperm adenylyl cyclase system is unique and regulated differently from that of other cells.

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